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(54) Method for conversion of a starch material, and enzyme composition suitable therefor

(57) Conversion of a starch material utilizing a thermostable non-reducing glucan-forming enzyme activity (NRGFE), for instance from *Sulfolobus solfataricus*. Native starch is depolymerized at 60-120°C and pH 4-9 in the presence of a thermostable NRGFE by means of thermostable glucanases, such as α -amylases, isoamylases, pullulanases and/or amylopullulanases, with formation of Maillard products having been suppressed by virtue of the NRGFE activity present. The non-reducing

glucans obtained can be used for the formation of trehalose by treating them with glucanases such as amylases, glucosidases and/or amyloglucosidases. Also, they can be modified with the aid of glucosyltransferases, for instance be treated with a thermostable phosphorylase in the presence of a glucosyl donor to raise the degree of polymerization, or be derivatized chemically or otherwise, optionally followed by a new hydrolytic treatment.

EP 0 727 485 A1

Description

FIELD OF THE INVENTION

The invention lies in the field of starch technology and more particularly concerns methods for converting starch utilizing enzymes. Such methods are generally aimed at the preparation of derivatives of starch which have a modified degree of polymerization.

The invention relates in particular to an enzymatic method for the preparation of starch derivatives having a modified degree of polymerization with respect to the starting starch, with the non-reducing character of the starting starch being substantially maintained.

BACKGROUND OF THE INVENTION

On the basis of existing knowledge in the state of the art, it is possible, by converting starch into cyclomaltodextrins using cyclomaltodextrin glucanotransferases (EC 2.4.1.19), to obtain products in which the non-reducing character of the starting material has been maintained. The cyclic products formed, however, have different properties, such as a reduced solubility and capacity to complex, than non-cyclic glucans.

The formation of non-cyclic non-reducing glucans has heretofore been found possible by converting starch hydrolysates with a reducing character at temperatures below 60°C into preparations with a non-reducing character. For the formation of non-reducing saccharides, thermolabile enzymes from mesophilic microorganisms are used, as described in European patent publication EP 0 606 753 A2. One of the enzymes is a non-reducing saccharide-forming enzyme, which catalyzes the formation of non-reducing saccharides ending with a trehalose structure. Enzymes of this type will herein be designated as non-reducing glucan (NRG) forming enzymes.

The method according to EP 0 606 753 A2 starts from a starch syrup with a reduced degree of polymerization, which has been obtained by hydrolysis of gelatinized and liquefied starch at temperatures above 60°C according to generally known methods. In this syrup preparation, the reducing character of the starch increases, as does the formation of colored components which are a result of Maillard reactions. The undesired presence of such reaction products, formed by the reaction of aldoses with amino groups, means that in the production of the syrups it is preferred to use starting material that is free from proteins, peptides and amino acids, and also that Maillard products and material with still free amino groups, present in the syrups obtained, have to be removed by generally known methods to prevent further Maillardation during drying.

A method in which starch, optionally directly in a one-step process, can be converted into non-reducing products with a modified degree of polymerization would therefore constitute an important improvement.

It might be tried to realize such a direct conversion of starch into non-reducing products by combining the thermolabile enzymes according to EP 0 606 753 A2, which are only active at temperatures lower than 60°C, with hydrolytic enzymes capable of decomposing starch below the gelatinizing temperature. Amylases capable of doing this are for instance described in an article by Wijbenga et al. (Appl. Microbiol. Biotechnol. 35 (1991) 180-184). However, the most important reaction product here is glucose, which cannot serve as starting material for NRG-forming enzymes from patent specification EP 0 606 753 A2.

A different approach might consist in decomposition with thermostable enzymes active above 60°C, enabling work above the gelatinizing temperature of starch. Hydrolytic enzymes, such as α -, β - and glucoamylases, which are capable of doing so, are commercially available and find extensive application in the industry.

Advantages of conducting such a process at higher temperatures are: reduced chance of microbial contamination, increased reaction rate and a lower viscosity enabling high substrate concentrations. The combination of such thermostable hydrolases with an NRG-forming enzyme (NRGFE) thus has evident advantages. For such a method, however, an NRGFE with thermostable properties is indispensable. Such enzymes are not known, nor have any been found in the extensive screening of microorganisms described in EP 0 606 753 A2.

The above means that according to the present state of the art it is impossible to convert starch directly, i.e. in a one-step process, into non-reducing glucans.

Thermostable enzymes might be obtained by modification at a protein or DNA level of enzymes active at lower temperatures. Alternatively, they might be isolated from nature, in particular from (not necessarily thermophilic) organisms, which may belong to the domains of the *Archaea*, *Bacteria* and *Eukarya*.

Surprisingly, it has been established that thermostable enzymes with which such a method can be carried out can indeed be isolated from microorganisms belonging to the domain of the *Archaea*. Specifically, it has been found that a thermophilic archaeon belonging to the genus *Sulfolobus*, in particular *Sulfolobus solfataricus* DSM-1617, produces an enzyme which possesses the necessary properties for its use in a method that can be carried out above 60°C.

SUMMARY OF THE INVENTION

The invention relates to thermostable enzyme preparations comprising an NRG-forming entity that is catalytically active at temperatures higher than 60°C, so that reactions above the gelatinizing temperature of starch are possible.

The invention further relates to a method for converting starch, utilising a combination of thermostable enzymes including an NRG-forming entity, into products

having a modified degree of polymerization while the non-reducing character of the starch preparation is maintained.

As will be explained in more detail, the use of thermostable NRG-forming enzymes, in combination with thermostable starch-decomposing hydrolases, such as amylases, isoamylases, (amyllo)pullulanases as well as with transferases, enables the direct production from starch of non-cyclic glucans of different chain length, with the non-reducing character of the starting material being maintained.

As starting material in the method according to the invention, a starch material is used. The term starch material here denotes native starch and products with a different degree of polymerization derived therefrom. The starch material to be used can be of various origin, such as potato starch, maize starch, wheat starch, tapioca starch, pea starch, waxy-maize starch and other amylose-free or low-amylose (hence amylopectin-rich) starches, and high-amylose starch. It is also possible to use a modified starch which has been obtained by chemical, enzymatic and/or physical modification of native starch. The term starch material as used here also encompasses such modified starch products.

DETAILED DESCRIPTION OF THE INVENTION

The invention comprises a method for conversion of a starch material, characterized in that the conversion takes place utilizing a thermostable non-reducing glucan-forming enzyme activity (NRGFE). According to the invention, the conversion preferably occurs at 60-120°C, preferably 70-80°C, and at pH 4-9, preferably pH 5-7.

NRG-forming entity or enzyme activity is intended to refer in particular to enzymes capable of catalyzing the reaction $G_n \rightarrow G_{n-2}T$, wherein G represents a glucosyl residue, T a trehalosyl unit, and n an integer.

The thermostable NRGFE to be used according to the invention preferably originates from microorganisms, more specifically from *Sulfolobus solfataricus* DSM 1617 or a microorganism descending or derived therefrom. 'Originating from' and 'derived from' are here intended only to denote origin, and are specifically not intended as a limitation to 'produced by'. Thus the invention comprises the possibility that the enzyme is produced by a recombinant host different from the organism that produces the enzyme by nature.

Although modified and derivatized starch products can function as starting material, as has already been indicated above, it is preferred to use a native starch as starting starch material.

According to one embodiment of the invention, the starch material is depolymerized under simultaneous formation of non-reducing glucans. The depolymerization is preferably carried out with thermostable glucanases, such as in particular thermostable α -amylases, isoamylases, pullulanases, amylopullulanases or com-

binations thereof. Simultaneously with the depolymerization, non-reducing glucans are formed as a result of the catalytic action of the NRGFE. During the process operation and during subsequent processes, the formation of Maillard products is suppressed by virtue of the permanent absence of reducing terminal ends.

According to another embodiment of the invention, trehalose is formed from the non-reducing glucans by using suitable glucanases, in particular enzymes such as amylases, glucosidases, amyloglucosidases or combinations thereof. An enzyme useful for this purpose can for instance be found in *Sulfolobus solfataricus*. Useful enzymes for the release of trehalose from the NRG formed are described, for instance, in EP 0 628 630 A2.

According to yet another embodiment of the invention, the non-reducing glucans are modified by using glucosyltransferases. Thus the invention comprises, for instance, a method whereby the degree of polymerization of the non-reducing glucans is increased through the use of a phosphorylase in the presence of a glucosyl donor, such as glucose-1-phosphate. It is preferred to use a thermostable phosphorylase at 60-120°C. The thermostable phosphorylase has preferably been obtained from microorganisms belonging to the order of *Thermococcales*, the order of *Thermoproteales*, the genus *Bacillus*, the genus *Thermus* or the genus *Thermotoga*, or a microorganism descending or derived therefrom, more particularly originating from *Pyrococcus furiosus*, *Pyrococcus woesei*, *Thermoproteus tenax*, *Bacillus stearothermophilus*, *Bacillus caldolyticus*, *Thermus aquaticus*, *Thermus thermophilus*, *Thermus species ATCC 27737*, *Thermotoga maritima*, or a microorganism descending or derived therefrom. Here, too, it holds that according to the invention it is not necessary that an enzyme originating from a particular organism is in fact obtained from this organism; production through a recombinant host is expressly encompassed by the invention.

According to the invention, it is preferred to prepare non-reducing glucans that can be used in food, cosmetics, pharmaceutical or industrial products. For instance, non-reducing glucans are prepared which can be used as fat substitutes or sweetener in food and foodstuffs, or as stabilizer or as filler in food, cosmetics or pharmaceutical products.

The non-reducing glucans obtained can also be derivatized (e.g. chemically, physically or enzymatically), for instance to synthesize detergents. For that purpose, preferably the non-polar molecule tails of the detergent are coupled to (the ends of) the non-reducing glucans.

Formed derivatives, such as detergents, can be hydrolyzed again, for instance through a biocatalytic method or by the use of physicochemical methods. If thereby the α 1,4-bond between the trehalosyl unit and the adjacent glucosyl unit is hydrolyzed (for instance, into a compound of formula $XG_{n-2}TX$, wherein X represents a substituent and G, T and n have the aforementioned

meanings), this leads to the formation of a monosubstituted trehalosyl unit (for instance, a compound of formula TX). Such a monosubstituted trehalose compound is difficult to synthesize via a conventional route because trehalose is made up of two identical units. The monosubstituted trehalose compound can subsequently be further derivatized, e.g. into a trehalose unit substituted with different groups, having the formula YTX, wherein Y and X are different substituents.

Reducing derivatives, such as detergents, can also be prepared by hydrolysis of the 1,1-bond, either through a biocatalytic method, or with the aid of physicochemical methods.

The invention also comprises a method whereby the trehalose is further derivatized (e.g. physically, chemically or catalytically), for instance to prepare detergents, with the non-polar parts of the detergents being coupled to (the terminal ends of) the trehalose. In that case, too, it holds that reducing detergents on a carbohydrate basis can be prepared by hydrolysis of the 1,1-bond of trehalose, while the hydrolysis of the 1,1-bond can occur through a biocatalytic process or physicochemical methods.

The invention further comprises the non-reducing glucans and products derived therefrom, obtainable or obtained with the above-defined method. The invention also encompasses the use of non-reducing glucans and products derived therefrom as defined above, in food, cosmetics, pharmaceutical or industrial products, in particular as fat substitute or sweetener in foodstuffs, as stabilizer or as filler in foodstuffs, cosmetics and pharmaceutical products, or as detergents.

The invention is also embodied in a thermostable non-reducing glucan-forming enzyme active at a temperature of 60-120°C, preferably 70-80°C. In particular, the invention comprises thermostable NRGFE originating from *Sulfolobus solfataricus* DSM 1617 or a microorganism descending or derived therefrom. The invention also provides an enzyme preparation comprising a thermostable non-reducing glucan-forming enzyme (NRGFE) as defined above, in combination with an enzyme selected from the group consisting of α -amylases, isoamylases, pullulanases, amylopullulanases, amylases, glucosidases, amyloglucosidases, and glucosyltransferases (for instance phosphorylases). Preferably, all enzymes present in such an enzyme preparation are thermostable.

A thermostable enzyme to be used according to the invention can be obtained in the following manner.

Medium and fermentation conditions

Sulfolobus solfataricus DSM-1617 could be cultured in a suitable medium thereof, such as that consisting of the following constituents: yeast extract (2.0 g/l), KH_2PO_4 (3.1 g/l), $(\text{NH}_4)_2\text{SO}_4$ (2.5 g/l), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/l), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.25 g/l) and trace elements (0.0018 g/l $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.0045 g/l $\text{Na}_2\text{B}_4\text{O}_7$.

$10\text{H}_2\text{O}$, $0.22 \cdot 10^{-3}$ g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $0.05 \cdot 10^{-3}$ g/l $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $0.03 \cdot 10^{-3}$ g/l $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $0.03 \cdot 10^{-3}$ g/l $\text{VOSO}_4 \cdot 2\text{H}_2\text{O}$ and $0.01 \cdot 10^{-3}$ g/l $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$). With 0.1 M H_2SO_4 the pH was adjusted to 3.5.

Volumes of 5 liters of medium were sterilized in fermentors of a volume of 7 liters (Applikon, Schiedam, Netherlands) by autoclaving for 45 minutes at 120°C and 1.2 bar. Precultures were cultured in 250 ml Erlenmeyer flasks with 70 ml medium (autoclaved for 20 minutes at 120°C and 1.2 bar) at 80°C and 150 rpm in a shaking water bath. The fermentors were inoculated with 280 ml preculture. Growth in the fermentors occurred at 80°C with an aeration of 0.35 liter air/liter medium/minute at a stirring speed of 400 rpm. Growth was monitored by measuring the optical density of the culture at 550 nm and 1 cm path length. At the beginning of the stationary phase with a fermentation period of about 40 hours, the fermentation can be stopped. By centrifugation cells could be separated from the culture liquid.

It is possible to recover NRGFE from cells and from cell-free culture liquid. In doing so, one can use existing technologies for concentration, such as evaporation, ammonium sulfate precipitation, acetone precipitation and membrane filtration, and for purification, such as ion exchange chromatography, gel filtration, isoelectric focussing, hydrophobic interaction chromatography, affinity-chromatography and preparative electrophoresis.

Thus NRGFE could be recovered from cell extracts. To that end, cells were included in 10 mM Tris-HCl buffer (3 ml/g cells wet weight) and subsequently disintegrated at 0°C using ultrasound (disintegrator Soniprep 150, MSE). After centrifugation at 6°C and 35000 g for 60 minutes, the crude cell-free extract was dialyzed against 0.05 M acetate buffer pH 5.5. A crude enzyme preparation was subsequently obtained after 65% ammonium sulfate precipitation followed by acetone precipitation with two volume units of cold acetone. After centrifugation at 6°C and 5000 g for 10 minutes, the precipitated proteins were resuspended in 0.05 M acetate buffer pH 5.5 and dialyzed against this buffer at 6°C.

The thus obtained crude enzyme preparation could be further purified through gel filtration at 6°C with a Hi-load 26/60 Superdex column (Pharmacia Biotech AB, Uppsala, Sweden).

The chromatographic conditions were as follows: column volume: 325 ml; elution rate: 1 ml/min; eluent: 0.05 M acetate buffer, 0.15 M NaCl pH 5.5; fraction volume: 5.5 ml; applied: 10 ml crude enzyme preparation.

The following analyses were performed.

Protein

The elution was monitored by measuring the absorption at 280 nm. Protein concentrations were determined according to the Bradford method with a kit of Bio-Rad and BSA (bovine serum albumin) as standard.

Oligosaccharide composition of reaction products

The oligosaccharide composition was determined by HPLC. HPLC with a cation exchanger was performed with Benson BCX4 cation exchanger in the calcium form (particle size 15-20 μm , column 250 x 10 mm), with 100 ppm Ca-EDTA as mobile phase, an elution rate of 0.4 ml/min, a column temperature of 85°C and a sample size of 20 μl . The refractive index of the eluted material was determined with a Jasco 830-RI detector at 40°C and registered with an SP4400 Chromjet integrator of Spectra Physics at a paper rate of 2.5 mm/min. HPLC with an anion exchanger was carried out with a Dionex CarboPac PA1 column (250 x 4 mm) with a CarboPac PA1 guard column. Detection occurred with a Dionex PAD II pulsed amperometric detector of a sensitivity of 1 μC . The elution rate was 1 ml/min, pressure 90 bar and the sample volume 20 μl (150 μl preflush). Used as mobile phase was 0.1 M NaOH (eluent A) with a gradient of 0-0.6 M sodium acetate (eluent B) in the following manner: %B in eluent; 5% (0 min) - 35% (10 min) - 60% (20 min) - 100% (25-27 min) - 5% (28-35 min).

NRG-forming activity

Substrate: 0.6% soluble starch (Merck) from which the α 1,6 bonds had been removed using isoamylase (Hayashibara Biochem Lab, Japan), dissolved in buffer; enzyme/substrate ratio: 0.5 ml enzyme + 6 ml substrate; temperature: 70°C; reaction time: 18.5 hours; buffer: 0.05 M sodium acetate buffer pH 5.5; analysis: decrease of reducing end groups according to Nelson-Somogyi; activity expressed in units per ml with 1 unit (U) being equal to the decrease of 1 μmol reducing power per minute (amylase activity could be determined in the same manner as the increase of reducing end groups).

Thus one peak with NRG-forming activity obtained eluting between 71 and 138 ml after beginning of the void peak; according to a calibration line drawn up with ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), β -amylase (200 kDa) and apoferritin (443 kDa) corresponding with a molecular weight of about $5 \cdot 10^4$ Da. The active fractions from a number of gel filtrations were collected. After dialysis against 20 mM piperazine pH 5.5 with 0.02% NaN_3 , this material was further purified by means of anion exchange chromatography with a Mono Q HR 10/10 FPLC column (Pharmacia Biotech AB, Uppsala, Sweden).

The chromatographic conditions were as follows: column volume: 8 ml; elution rate: 2 ml/min; eluent: 20 mM piperazine pH 5.5; gradient: 0-0.5 M NaCl in eluent in 45 min followed by 0.5-1M NaCl in 25 min and 1 M NaCl for 20 min; fraction volume: 60 ml during application, 2 ml during gradient-elution; volume applied: 867 ml with 38 mg protein.

Assays were carried out as described hereinbefore, with NRG-forming activity being determined with 0.2 ml

enzyme and with 4 ml 0.6% maltooligosaccharides having an average degree of polymerization of approx. 6 (DP6) as substrate (according to HPLC containing maltopentaose, maltohexaose and maltoheptaose in the ratio 1:3:1.4).

Thus NRG-forming activity, which appeared present in the run-through with 85% of the protein applied, could be separated from an amylolytic activity, bound to the column material under these conditions, which eluted between 14 and 27 min after start of the gradient.

Material present in the run-through of a number of anion exchange chromatographies performed as described above, was, after dialysis against 0.02 M Tris/HCl pH 7.5 + 0.02% NaN_3 , further purified by means of anion exchange chromatography with a Mono Q HR 10/10 column under the following conditions: column volume: 8 ml; elution rate: 1 ml/min; eluent: 0.02 M Tris/HCl pH 7.5; gradient: 0-0.075 M NaCl in eluent in 5 min followed by 0.075-0.225 M NaCl in 60 min, 0.225-0.5 M NaCl in 30 min and 0.5 M NaCl for 25 min; fraction volume: 59 ml during application, 1 ml during gradient elution; volume applied: 1450 ml with 46 mg protein.

Assays were performed as described hereinbefore, with NRG-forming activity being determined with 0.025 ml enzyme and 4 ml 0.6% DP6.

In this way NRG-forming activity eluted between 12 and 35 minutes after start of the gradient. This material was subjected to a dialysis against Milli-Q water. A purified NRGFE useful in the method could thereafter be obtained by multiple separation using the Rotofor isoelectric focusing system of Bio-Rad under the following conditions: applied volume first run: 10 ml dialyzed sample containing 5.5 mg protein (15 mg based on A280 BSA 1 mg/ml=0.68) with 45 ml Milli-Q water, 2.6 ml Ampholine 3.5-10 (Pharmacia Biotech AB, Uppsala, Sweden) and urea in a final concentration of 3 M; power: 12 W, constant; run time: 4 hours; fractions: 20 with a volume of 2.5 ml.

The pH in the fractions rose from 4.03 to 10.78. The NRG-forming activity was determined after dialysis against 0.02 M Tris/HCl pH 7.5 with 0.25 ml enzyme and 4 ml 0.6% DP6. Thus activity was found between fractions with pH 4.66 and 7.22.

With this material a second run was carried out, in which the following variables were used: applied volume second run: 8 ml of fractions from the first run focussing between pH 4.66 and 7.22 containing 1.5 mg (4.7 mg protein based on A280 BSA 1 mg/ml=0.68), with 49 ml Milli-Q water and urea in a final concentration of 3 M; power: 12 W, constant; run time: 3 hours; fractions: 20 with volume of 2.5 ml.

The pH in the fractions rose from 4.05 to 11.87. The NRG-forming activity after dialysis against 0.02 M Tris/HCl pH 7.5 was determined with 0.15 ml enzyme and 4 ml 0.6% DP6 after 2 hours of incubation.

Thus one peak of activity was found with 1.4 mg protein, between fractions with pH 4.71 and 7.01 with

the top at pH 5.7.

For the thus obtained purified and dialyzed NRGFE preparation, the following properties were determined.

pH range

Substrate: 1.2% DP6; buffer: 0.1 M bis-tris-propane + 0.1 M citrate + 0.1 M maleate + 0.02% NaN_3 ; pH 3-9.5 rising in steps of 0.5; enzyme/substrate ratio: 0.2 ml substrate with 0.2 ml buffer of the desired pH and 0.005 ml NRGFE; reaction time: 17.5 hours; temperature: 70°C; analysis: change in reducing end groups according to Nelson-Somogyi.

Activity was found between pH 4.5 and 9.0 with an optimum at pH 6.0.

pH stability

Buffer: 0.1 M bis-tris-propane + 0.1 M citrate + 0.1 M maleate + 0.02% NaN_3 ; pH 3-9.5 with steps of 0.5 pH unit; enzyme: 0.05 ml NRGFE with 0.150 ml buffer of the desired pH; temperature: 21°C; reaction time: 1 hour; analysis: activity was determined as above at pH 6.0 in a mixture with the incubated enzyme mixture, 1.5 ml buffer pH 6.0 and 1.3 ml 1.4% DP6.

It was found that activity of NRGFE had been maintained with incubations performed at pH 5.5-9.5 and had disappeared after incubation at pH 3 and 3.5.

Temperature range

Substrate: 1.2% DP6 in buffer; buffer: 0.1 M bis-tris-propane + 0.1 M citrate + 0.1 M maleate + 0.02% NaN_3 ; pH 6.0; enzyme/substrate ratio: 0.15 ml substrate with 0.15 ml NRGFE; reaction time: 0.5 hours; temperature: 50-100°C, in steps of 5°C; analysis: change in reducing end groups.

Activity was observed in the entire temperature range with an optimum at 75°C.

Temperature stability

Buffer: 0.1 M bis-tris-propane + 0.1 M citrate + 0.1 M maleate + 0.02% NaN_3 ; pH 6; enzyme: 0.1 ml NRGFE; temperature: 75-100°C, in steps of 5°C; reaction time: 0.5 and 3.5 hours; analysis: activity was determined as above at pH 6.0 with 0.1 ml of the incubated enzyme mixture and 0.1 ml buffer with 1.2% DP6 for 4 hours at 70°C.

In this way it was found that activity had been maintained with incubations performed at 75-90°C; after 0.5 hours at 95°C there still remained 94% of the activity of the NRGFE and after 3.5 hours still 47%. After 0.5 hours at 100°C there was 1% activity left.

Characteristic of the action of the NRGFE is the possibility of forming non-reducing glucans from reducing substrates such as DP6 at temperatures above 60°C.

Production of non-reducing glucans from DP6

Substrate: 1.2% DP6 in buffer; buffer: 0.1 M bis-tris-propane + 0.1 M citrate + 0.1 M maleate + 0.02% NaN_3 ; pH 6; enzyme/substrate ratio: 4 ml NRGFE with 4 ml substrate; reaction time: 18 hours; temperature: 70°C; analysis: change in reducing end groups; HPLC.

Thus the amount of reducing end groups was reduced by 68%. The disappearance of reducing power was found to be accompanied by the appearance, on chromatograms which had been obtained after HPLC with anion exchanger, of new peaks with retention times of 6.5, 7.7 and 8.8 minutes next to those of maltopentaose, maltohexaose and maltoheptaose with retention times of 10.4, 11.5 and 12.4 minutes.

These peaks disappeared after incubation of 1.5 ml of the reaction mixture with 1 ml of a solution with 1.05 mg/ml amyloglucosidase (*Aspergillus niger*, 75 u/mg; Merck, Darmstadt, Germany) in 0.05 M maleate buffer pH 5.0 for 21 hours at 37°C. After this treatment it was found that peaks were formed with retention times of 2.2 and 3.0 min, corresponding with those of α , α -trehalose and glucose. From HPLC with cation exchanger a mass ratio of 1:3.4 could be determined for the di- and monosaccharide. After treatment with a solution of amyloglucosidase in combination with 0.032 mg/ml trehalase (pig kidney, 0.68 u/mg; Sigma, St. Louis, USA), solely a peak with a retention time of 3.0 min was found to be formed.

From this it could be concluded that through NRGFE, reducing glucans of the overall formula G_n , wherein G stands for a glucosyl residue and subscript n for an integer are converted into non-reducing glucans of the overall formula $G_{n-2}T$ wherein T stands for a trehalosyl unit.

A characteristic property of preparations containing non-reducing glucans is decrease of coloration resulting from the Maillard reaction.

Maillard reaction

Formation of non-reducing glucans-containing DP6 preparation: 3 ml 2% DP6 in 0.1 M acetate buffer pH 5.7 + 4 mM calcium chloride + 0.04% NaN_3 was incubated with 3 ml NRGFE at 80°C for 18 hours. After incubation the amount of reducing end groups was found to have decreased by 51% compared with similarly treated DP6 that had not been incubated with NRGFE but with 3 ml 0.02 M Tris/HCl.

Reaction: 2 ml of the above liquids + 0.4 ml 10% glycine + 1.6 ml 2.2 mM NaOH; pH 7.1; reaction time: 17 hours; temperature: 95°C; analysis: increase absorption at 450 nm.

In this way, with DP6 an increase of the absorption from 0.005 to 0.164 was established and with the non-reducing-glucans-containing DP6 an increase from 0.008 to 0.045.

The method according to the invention can be carried out with NRGFE produced by *Sulfolobus solfataricus*.

cus DSM-1617, but any other organism that produces an NRGFE active above 60°C can also be used. After culturing such organisms in a manner suitable therefor, NRGFE containing sources, such as cells, cell extracts or cell-free culture liquids, can as such be used in the method. Optionally, prior to use, concentration by means of existing technologies, such as centrifugation and filtration, can take place. In cases where components such as hydrolytic activities present in these enzyme sources constitute an impediment to the practice of the method, such an activity can be suppressed, for instance by the use of inhibitors, or the NRGFE can be purified. As described hereinabove for the NRGFE originating from *Sulfolobus solfataricus* DSM-1617, existing techniques can be used for this purpose.

NRG-forming enzyme preparations can be used in immobilized form, so that they can be simply separated from the reaction mixture. This also applies to the enzymes in the combination with which the method according to the invention is carried out.

In enzyme combinations which are used in the method according to the invention, in addition to NRGFE, in principle any enzyme with thermostable properties can be used and preferably enzymes belonging to the class of oxidoreductases, hydrolases, transferases, lyases or isomerases including those enzymes belonging to the subclasses EC 1.1, EC 2.4, EC 2.7, EC 3.1, EC 3.2, EC 4.2, EC 5.1, EC 5.3, EC 5.4 and in particular those forming part of the sub-subclasses EC 1.1.3, EC 2.4.1, EC 2.7.1, EC 3.1.3, EC 3.2.1, EC 4.2.2, EC 5.1.3, EC 5.3.1, EC 5.4.1, EC 5.4.2, with enzymes that are active with starch as substrate being especially of importance.

The composition of the thermostable enzyme combination in the method is determinative of the composition of the products eventually obtained. A person skilled in the art can influence the product composition through a directed choice of the enzymes in combination with which the NRGFE is used. Thus, from starch, by the use of a hydrolase such as an isoamylase (EC 3.2.1.68) or a pullulanase (EC 3.2.1.41), oligosaccharides having a degree of polymerization (DP) greater than 5 can be obtained, which can serve as substrate for the NRGFE used in combination. By the use of the method in this manner, it is possible to obtain a product consisting entirely of non-reducing glucans. When using other enzymes in the combination, specifically hydrolases such as α -amylase (EC 3.2.1.1), β -amylase (EC 3.2.1.2), amyloglucosidase (EC 3.2.1.3), α -glucosidase (EC 3.2.1.20), amylo-pullulanase or transferases such as amyloamylase (EC 2.4.1.25) or cyclomaltodextrin glucanotransferases (EC 2.4.1.19), products with a low DP, such as glucose, can be formed, which cannot be used as substrate by the NRGFE present in the combination. To separate such reaction products from the starting materials, proven technologies such as membrane filtration or batchwise feed/dilution of, respectively, the enzyme combination/substrate and product can be em-

ployed. Reactions in two-phase systems can also be employed.

By separating starting materials from reaction products, also an efficiency improvement can arise as a result of the elimination of final product inhibition.

The possibilities of separating undesired reaction products can be improved through specific derivatization of such a component. Thus, by the addition of glucose oxidase (EC 1.1.3.4) glucose can be converted into gluconic acid, which reaction product can be selectively removed through existing technologies.

In the method according to the invention, enzymes can be added which can use the non-reducing glucans formed as substrate, such as hexosyltransferases (EC 2.4.1), in particular the glucosyltransferases such as 4- α -glucanotransferase (EC 2.4.1.25) and trehalose phosphorylase (EC 2.4.1.64) and especially phosphorylase (EC 2.4.1.1) which, depending on reaction conditions, in particular the concentration of inorganic phosphate (P_i), can effect the transfer of a glucosyl unit from or to a non-reducing end of a glucan. In Example 3 this is demonstrated for phosphorylase from potato.

Research performed by applicant, inter alia, has shown that thermostable phosphorylases occur in the domains of *Archaea*, *Bacteria* and *Eukarya*, specifically in microorganisms belonging to the order of *Thermococcales*, the order of *Thermoproteales*, the genus *Bacillus*, the genus *Thermus* or the genus *Thermotoga*, viz. *Pyrococcus furiosus*, *Pyrococcus woesei*, *Thermoproteus tenax*, *Bacillus stearothermophilus*, *Bacillus caldolyticus*, *Thermus aquaticus*, *Thermus thermophilus*, *Thermus species* ATCC 27737 and *Thermotoga maritima*.

Depending on the result contemplated, at a time to be determined by the skilled person, the enzyme combination can be changed in composition, for instance by using immobilized enzymes, while, if necessary, the reaction conditions can be adjusted.

With the method according to the invention, the person skilled in the art, through an appropriate combination of enzymes, can obtain as product non-reducing glucans or products derived therefrom with a degree of polymerization of 2 and higher.

In cases where it is desirable not to add non-reducing glucans beforehand to the products to be prepared, the method according to the invention can be practised *in situ*, an example of a case in point being the preparation of starch containing bakery products where temperatures above 60°C are employed, such as bread-making.

The method according to the invention is carried out at temperatures above 60°C with starch material as substrate. The starch material can be present in aqueous but also in a water-limited environment. An aqueous solution of the starch material, preferably with a starch concentration between 0.1 and 50 percent by weight, is obtained by heating above the gelatinizing temperature of the starch material used or by dissolving pregelatinized starch material in water.

The method according to the invention is carried out with a combination of enzymes, and the person skilled in the art can determine an optimum combination of conditions for use of the desired combination of enzymes on the basis of known data about the individual enzymes, inter alia with regard to pH, temperature and substrate dependency.

When using the NRGFE originating from *Sulfolobus solfataricus* DSM-1617, the method can be carried out at a temperature between 60 and 120°C, preferably between 70 and 80°C. The pH values when using NRGFE from *Sulfolobus solfataricus* DSM-1617 can vary between 4.5 and 9 and preferably between pH 5 and 7. The reaction time is not limited but is preferably between 1 and 100 hours.

The NRG-containing products that are formed with the method according to the invention can be used as such or be stripped of impurities and concentrated with known technologies such as centrifugation, filtration, reverse osmosis and evaporation. If necessary, reducing glucans and other undesired components such as salts can be removed with techniques available therefor. Results described above with HPLC with anion exchanger indicate that ion exchange chromatography can for instance be used to separate the different glucans from each other.

The NRG obtained can be used in methods where starch material is used and where the absence of reducing power is of importance. Applications of starch and starch derivatives are for instance found in the paper, corrugated board, adhesive, detergent, textile and food-stuff industries, in the cosmetic and pharmaceutical industries, and in the oil and gas extraction industry. Such applications are described inter alia by R.J. Alexander in "Maltodextrins: Production, Properties and Applications" (in: Starch hydrolysis products: worldwide technology, production and application. F.W. Schenck, F.E. Hebeda eds., VCH Publishers, New York, 1992, pp. 233-275). Without wishing to be complete, they are methods involving application as fat substitute, sweetener, stabilizer, filler, spray drying additive, adsorbent for flavors, fabric softener, bleach activator, surfactant, emulsifier (detergent), complexing agent, sequestering agent and in coatings and drilling muds. The use of NRG prevents reactions which may involve the reducing end of starch, such as they may, for instance, occur with oxygen, with amino groups of amino acids, peptides and proteins present in products, or with fine chemicals present in the products, such as flavors. NRGs could therefore serve as substitutes of hydrogenated or oxidized starch materials.

The above-mentioned stability is also of advantage when using NRGs instead of starch material as starting material for derivatization reactions which are generally carried out under acid or basic conditions and/or at elevated temperatures. For instance in etherification reactions of starch materials in alkaline environment, the use of NRGs will involve a highly reduced browning.

In the NRG formed through the method according to the invention, an additional non-reducing end has been formed which can be used for further derivatization. This can for instance occur in a continuous process wherein the method according to the invention is carried out in a membrane reactor with an amylase and NRGFE as enzyme combination. The formed NRG with a low DP is thereby removed and can be derivatized in a next compartment. Such a derivatization can be a coupling of non-polar tails to non-reducing ends, so that detergents are formed.

In the NRG obtained by means of the method according to the invention a trehalosyl unit has been introduced through NRGFE.

The NRG formed on the basis of starch by means of the method according to the invention and the substances derived therefrom contain a trehalosyl unit which is characterized by a specific type of bond. The glucan is thus provided with a bond unique to the glucan. In addition to the possibilities arising from the absence of the reducing end, this new bond opens additional possibilities for methods where the NRG is used. Thus this new bond provides the possibility of targetted hydrolysis of NRG and substances derived therefrom. For this purpose, physical, chemical and enzymatic methods can be used, such as the use of NRGFE under conditions where a reversal of the reaction described in this patent specification occurs. Conditions for practising such a method can be determined by a person skilled in the art and may mean that the reaction is carried out in a water-limited environment.

Further, for a person skilled in the art it is possible, by the use of biocatalytic, physical or chemical methods, to hydrolyze the α 1,4-bond between the trehalosyl unit and the adjacent glucosyl unit, which, for substances derived from NRG, can lead to the formation of a monosubstituted trehalosyl unit. Such a hydrolysis could be carried out biocatalytically, for instance by the use of an α -amylase optionally in combination with α -glucosidase or amyloglucosidase.

The invention thus provides the possibility of preparing non-reducing glucans by enzymatic route, which by their specific properties signify an extension or improvement of the possibility of using starch products and which can serve as a basis for further derivatization whereby new products can be obtained and/or on which new methods can be based.

The invention is explained in and by the following examples.

Example 1

In 8 fermentations of *Sulfolobus solfataricus* DSM-1617 under conditions described hereinbefore, with cells being harvested at an OD₅₅₀ between 0.54 and 0.66, an average cell yield of 1.52 g/l wet weight was obtained. Cells were separated from culture liquid by centrifugation. From the cells, in the manner described

hereinbefore, a purified enzyme preparation was obtained with 18.6 µg protein per ml and a specific activity of 2 U/mg. Assays took place as described hereinbefore.

The purified NRGFE containing preparation was used in a method in which potato starch was hydrolyzed with a commercial α-amylase.

2.5 ml of the purified NRGFE preparation was mixed with 2.5 ml of a solution with 0.4 µl Termamyl 120L (Novo Nordisk, Copenhagen, Denmark) per liter 0.1 M acetate buffer pH 5.7 + 4 mM CaCl₂ + 0.04 % NaN₃ and 5 ml 1.2% native potato starch (85% dry solids; AVEBE, Foxhol, Netherlands) in the same buffer. The reaction mixture was incubated at 80°C and after 24 and 48 hours 2 ml of sample was taken which, to stop the reaction, was set in ice water and was adjusted to pH 8.0 with 50 µl 1M Tris/HCl pH 8.5. In the samples the number of reducing end groups was determined and, by means of HPLC with cation exchanger, the content of products with a DP1-7, this as a measure for the decomposition of the starch by the α-amylase. The reducing power, expressed as dextrose equivalent (DE), was 6.9 after 24 hours and 11.1 after 48 hours. The content of DP1-7 was, respectively, 3.1 and 3.5 g/l, corresponding with 62 and 68% breakdown. HPLC with anion exchanger further showed the presence of products with retention times corresponding with those of non-reducing glucans. For comparison, the same reaction was carried out with 2.5 ml buffer without NRGFE. After 24 hours the DE in this reaction mixture was 11.2 and after 48 hours 18.5. The content of DP1-7 was, respectively, 2.5 and 3.3 g/l, corresponding with 51 and 65% decomposition of the native starch. HPLC with anion exchanger samples gave no indication of the presence of non-reducing glucans in this sample. So, given an equal decomposition, by the use of an enzyme combination with NRGFE the reducing power has remained considerably lower.

Example 2

The purified NRGFE preparation from Example 1 was used in combination with a commercial pullulanase.

A solution with 0.72 % soluble starch (90% dry solids; Merck, Darmstadt, Germany) in 0.1 M acetate buffer pH 5.7 + 4 mM CaCl₂ + 0.04 % NaN₃ was gelatinized by heating at 100°C for 15 minutes. After cooling to 60°C there was added to 5 ml of this solution 0.4 ml of the purified NRGFE preparation and 0.6 ml of a solution with 40 µl Promozyme 200L (Novo Nordisk, Copenhagen, Denmark) per liter in buffer. A similar incubation was performed without NRGFE. After 16.5 hours incubation of the reaction mixtures at 60°C the DE was determined. Without NRGFE it was found to have increased from 0.91 to 0.98 by the action of Promozyme. Combination of Promozyme with NRGFE resulted in a DE of 0.87, and the presence in this sample of products with a retention time corresponding with that of non-reducing

glucans could be demonstrated with the aid of HPLC with anion exchanger.

Example 3

A reaction mixture consisting of 3 ml 0.1 M acetate buffer + 4 mM CaCl₂ + 0.04% NaN₃ with 2% DP6 and 3 ml of the purified NRGFE preparation was incubated at 80°C for 18 hours. The number of reducing ends in this reaction mixture was found to have decreased by 51% compared with an incubation carried out without NRGFE. Both products, DP6 and NRG-containing DP6, were used as substrate for phosphorylase recovered from potato according to known methods. Reaction mixtures consisting of 0.375 ml of the above mixtures, with an activity of 2 units per ml, 2.5 ml phosphorylase (2 units per ml) and 5 ml 3% α-glucose-1-phosphate (G-1-P) in 0.05 M bis-tris-propane pH 7 were incubated at 35°C for 24 hours. The decrease in G-1-P calculated from the amount of inorganic phosphate formed, determined with the method of Chen, was found to be the same in both reaction mixtures (40%). The amount of polymeric material formed, determined with the anthrone-sulfuric acid method, after dialysis of 2 ml of the reaction mixtures against 0.1 M bis-tris-propane pH 7, was also found to be the same. After determination of the reducing power of the polymeric material from the two reaction mixtures, a DP of 50 could be calculated for the polymer from the reaction mixture with DP6, and a DP of 75 for the reaction mixture with NRG-containing DP6. To demonstrate that NRG had been used as primer for the polymer formed, the solutions obtained upon dialysis were treated with amyloglucosidase by incubating 0.2 ml of the dialyzed solutions with 0.4 ml amyloglucosidase (1 mg/ml; *Aspergillus niger*, 75 u/mg; Merck, Darmstadt, Germany) in 0.1 M acetate buffer pH 4.1 at 35°C for 15 hours. After incubation, with HPLC with anion exchanger no trehalose was demonstrable in polymer obtained with DP6, but it was in that obtained with NRG-containing DP6 as primer.

Claims

1. A method for conversion of a starch material, characterized in that the conversion takes place utilizing a thermostable non-reducing glucan-forming enzyme activity (NRGFE).
2. A method according to claim 1, characterized in that the conversion takes place at 60-120°C, preferably 70-80°C.
3. A method according to claim 1 or 2, characterized in that the conversion takes place at pH 4-9, preferably pH 5-7.
4. A method according to any one of claims 1-3, char-

acterized in that the thermostable NRGFE originates from microorganisms.

5. A method according to claim 4, characterized in that the thermostable NRGFE originates from *Sulfolobus solfataricus* DSM 1617 or a microorganism descending or derived therefrom. 5
6. A method according to any one of claims 1-5, characterized in that as starch material a native starch is used. 10
7. A method according to any one of claims 1-6, characterized in that the starch material is depolymerized under simultaneous formation of non-reducing glucans. 15
8. A method according to claim 7, characterized in that the depolymerization is carried out with the aid of thermostable glucanases, in particular thermostable α -amylases, isoamylases, pullulanases, amylopullulanases or combinations thereof. 20
9. A method according to any one of claims 1-8, characterized in that by the use of glucanases, such as amylases, glucosidases, amyloglucosidases or combinations thereof, trehalose is formed from the non-reducing glucans. 25
10. A method according to any one of claims 1-9, characterized in that the non-reducing glucans are modified using glucosyltransferases. 30
11. A method according to claim 10, characterized in that by the use of phosphorylase in the presence of a glucosyl donor, such as glucose-1-phosphate, the degree of polymerization of the non-reducing glucans is raised. 35
12. A method according to claim 11, characterized in that a thermostable phosphorylase is used at 60-120°C. 40
13. A method according to claim 12, characterized in that a thermostable phosphorylase is used which has been obtained from microorganisms belonging to the order of *Thermococcales*, the order of *Thermoproteales*, the genus *Bacillus*, the genus *Thermus* or the genus *Thermotoga*, or a microorganism descending or derived therefrom. 45 50
14. A method according to claim 13, characterized in that a thermostable phosphorylase is used which originates from *Pyrococcus furiosus*, *Pyrococcus woesei*, *Thermoproteus tenax*, *Bacillus stearothermophilus*, *Bacillus caldolyticus*, *Thermus aquaticus*, *Thermus thermophilus*, *Thermus species* ATCC 27737, *Thermotoga maritima*, or a microor-

ganism descending or derived therefrom.

15. A method according to any one of claims 1-14, characterized in that non-reducing glucans are prepared which can be used in food, cosmetics, pharmaceutical or industrial products.
16. A method according to claim 15, characterized in that non-reducing glucans are prepared, which can be used as fat substitutes or as sweetener in food and foodstuffs, or as stabilizer or as filler in food, cosmetics or pharmaceutical products.
17. A method according to any one of claims 1-16, characterized in that the non-reducing glucans are derivatized.
18. A method according to claim 17, characterized in that from the non-reducing glucans, detergents are synthesized.
19. A method according to claim 18, characterized in that of the detergent, the non-polar molecule tails are coupled to (the ends of) the non-reducing glucans.
20. A method according to any one of claims 17-19, characterized in that derivatized non-reducing glucans are hydrolyzed.
21. A method according to claim 20, characterized in that of the derivatized non-reducing glucans, the α 1,4-bond between the trehalosyl unit and the adjacent glucosyl unit is hydrolyzed, whereby a monosubstituted trehalose is obtained.
22. A method according to any one of claims 1-20, characterized in that reducing derivatives, such as detergents, are prepared through hydrolysis of the 1,1-bond.
23. A method according to claim 22, characterized in that the hydrolysis of the 1,1-bond is effected biocatalytically or with the aid of physicochemical methods.
24. A method according to claim 9 or 21, characterized in that the optionally substituted trehalose is derivatized.
25. A method according to claim 24, characterized in that on the basis of the trehalose, detergents are prepared.
26. A method according to claim 25, characterized in that of the detergent, the non-polar parts are coupled to (the ends of) the trehalos.

27. A method according to claim 26, characterized in that reducing detergents on a carbohydrate basis are prepared by means of hydrolysis of the 1,1-bond of trehalose. 5
28. A method according to claim 27, characterized in that the hydrolysis of the 1,1-bond occurs through a biocatalytic process or through physicochemical methods. 10
29. Non-reducing glucans and products derived therefrom, obtained or obtainable with the method according to any one of the preceding claims. 15
30. Use of non-reducing glucans and products derived therefrom as defined in claim 29, in food, cosmetics, pharmaceutical or industrial products, in particular as fat substitute or as sweetener in foodstuffs, as stabilizer or as filler in foodstuffs, cosmetics and pharmaceutical products, or as detergents. 20
31. Thermostable non-reducing glucan-forming enzyme which is active at a temperature of 60-120°C, preferably 70-80°C. 25
32. Enzyme according to claim 31, originating from *Sulfolobus solfataricus* DSM 1617 or a microorganism descending or derived therefrom. 30
33. Enzyme preparation comprising a thermostable non-reducing glucan-forming enzyme (NRGFE) as defined in claim 31 or 32, in combination with an enzyme selected from the group consisting of α -amylases, isoamylases, pullulanases, amylopullulanases, amylases, glucosidases, amyloglucosidases, and glucosyltransferases, such as phosphorylases. 35
34. Enzyme preparation according to claim 33, wherein each of the enzymes present therein is thermostable. 40
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EUROPEAN SEARCH REPORT

Application Number
EP 96 20 0401

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
X	BIOTECH. FORUM EUROPE, vol. 8, no. 4, 1991, pages 201-203, XP002003660 LAMA ET AL.: "Thermostable amylolytic activity from <i>Sulfolobus solfataricus</i> " * the whole document *	1-6, 15, 16, 29, 30	C12N9/24 C12N9/90 C12P19/24 C12P19/14 A61K31/70 A61K7/00
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A	---	33, 34	C12N C12P
A	EP-A-0 332 027 (NATIONAL STARCH AND CHEMICAL CORPORATION) 13 September 1989 * the whole document *	18, 19	
A	EP-A-0 499 434 (UNILEVER PLC) 19 August 1992 * page 5, line 34 - line 58 * * column 24 *	17-19, 24-26	
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 22 May 1996	Examiner Gac, G
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X: particularly relevant if taken alone Y: particularly relevant if combined with another document of the same category A: technological background : non-written disclosure P: intermediate document</p> <p>T: theory or principle underlying the invention E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons</p> <p> : member of the same patent family, corresponding document</p>			

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EUROPEAN SEARCH REPORT

Application Number
EP 96 20 0401

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	<p>DATABASE WPI Week 3285 Derwent Publications Ltd., London, GB; AN 193629 XP002003661 & JP-A-60 120 984 (IKEDA TOKA KOGYO) , 28 June 1985 * abstract *</p>	12-14	
P,X	<p>EP-A-0 688 866 (KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO) * page 2, line 50 - line 56 * * page 3 lines 12-23, line 56 * * page 4, line 53 - line 58 * * page 5 - page 7 * * page 11 experiment 3-2 * * page 12 experiment 4-2 *</p>	1-9, 15, 16, 29-34	
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 22 May 1996	Examiner Gac, 6
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<p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p>		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p>	

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